PAPER

The glucocorticoid receptor N363S polymorphism and steroid response in Duchenne dystrophy

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Background: Steroid administration is beneficial in Duchenne muscular dystrophy (DMD), but the response, incidence, and the severity of side effects are variable.
Aims: To investigate whether glucocorticoid receptor (*GRL*) gene polymorphisms may be responsible for glucocorticoid sensitivity in DMD.
Methods: Forty eight DMD patients treated either with prednisone or deflazacort were subjected to genetic analyses of the *GRL* gene.
Results: Mutation studies revealed an heterozygous A to G mutation at *GRL* cDNA position 1220 in three DMD patients resulting in an asparagine to serine amino acid change at amino acid position 363 (N363S). The N363S carrier DMD patients showed a trend towards a later age at loss of ambulation in comparison with non-carrier patients.
Conclusions: These data suggest that the N363S *GRL* polymorphism may be implicated in the long term response to glucocorticoids.

Duchenne muscular dystrophy (DMD) is a lethal childhood muscular disorder characterised clinically by progressive muscle wasting and weakness leading to loss of ambulation by a mean age of 10.5 years[1] and death due to respiratory or cardiac insufficiency in the early twenties.[2] To date, the only proven, effective pharmacological therapy in DMD is treatment with steroids,[3–11] but inter-patient variability in steroid response and severity of side effects are well known. Data derived from analysis of the standard deviation in several reports in the literature[7 9 12] often show standard deviations greater than the mean.[6 7 9 12 13] Moreover, wide variation in the age at which loss of ambulation occurs among steroid treated DMD patients is also reported.[13]

The mechanisms of action of prednisone in DMD are largely unknown. The well known anti-inflammatory/immunosuppressive action of prednisone alone does not explain the beneficial effect of glucocorticoids in DMD.[14] The activation of the intracellular glucocorticoid receptor (*GRL*) is an essential step in the glucocorticoid mediated signaling cascade. The *GRL* is a member of the intracellular steroid hormone binding superfamily of receptors.[15] It is a 94 kDa intracellular protein that in the bound state specifically binds and modulates the activity of target gene promoters. This interaction causes either stimulation or inhibition of transcription.[16]

To investigate if *GRL* polymorphisms may be associated with altered sensitivity to glucocorticoids we conducted genetic analyses of the *GRL* gene in a cohort of steroid treated DMD patients.

MATERIAL AND METHODS

Patients

A retrospective search of our DMD patient database was done for patients with molecularly proven DMD who were treated with prednisone or deflazacort for at least one year. Drug equivalence of 1:1.3 was calculated on the basis of previous studies.[17] Forty eight patients met the inclusion criteria.

Patient evaluation protocol

DMD patients were clinically evaluated during steroid treatment every three or four months by timed functional testing (gait and the ability to rise from a chair, climb stairs, and rise from the floor),[18] and by manual muscle testing using the Medical Research Council (MRC) scale in four muscles: deltoid, triceps, iliopsoas, and quadriceps. A composite functional score resulting from the sum of the grades in each single functional score was also calculated.[19] Age at the beginning of therapy, steroid therapy duration, and age at loss of ambulation were recorded.

Molecular studies

Polymerase chain reaction (PCR) amplification of the *GRL* gene and single strand conformational polymorphism (SSCP)/sequencing analysis of the PCR products were done as previously described.[20] *Tsp5091* restriction fragment length polymorphism analysis was performed in 48 DMD patients.

Statistical analysis

Z score was used to assess the probability of a patient to belong to the normal distribution of the age of loss of ambulation derived from our DMD patient population. The cumulative proportion of patients still ambulant was obtained using Kaplan-Meier curve. An analysis of the risk for loss of ambulation associated with selected independent variables (N363S polymorphism, age at beginning of therapy, and therapy duration) used the Cox proportional hazard model.

RESULTS

Patients

All 48 patients were enrolled in controlled clinical trials with either prednisone or deflazacort at our institution.[19 21] Prednisone was administrated at 0.75 mg/kg/day (13 patients) and deflazacort at a prednisone dose equivalent of 0.9 mg/kg/day (35 patients) for the first year of treatment, and then switched to an alternate day schedule with prednisone at 1.5 mg/kg and deflazacort at 1.8 mg/kg until loss of ambulation. All patients were ambulatory at entry in

Abbreviations: DMD, Duchenne muscular dystrophy; PCR, polymerase chain reaction; SSCP, single strand conformational polymorphism.

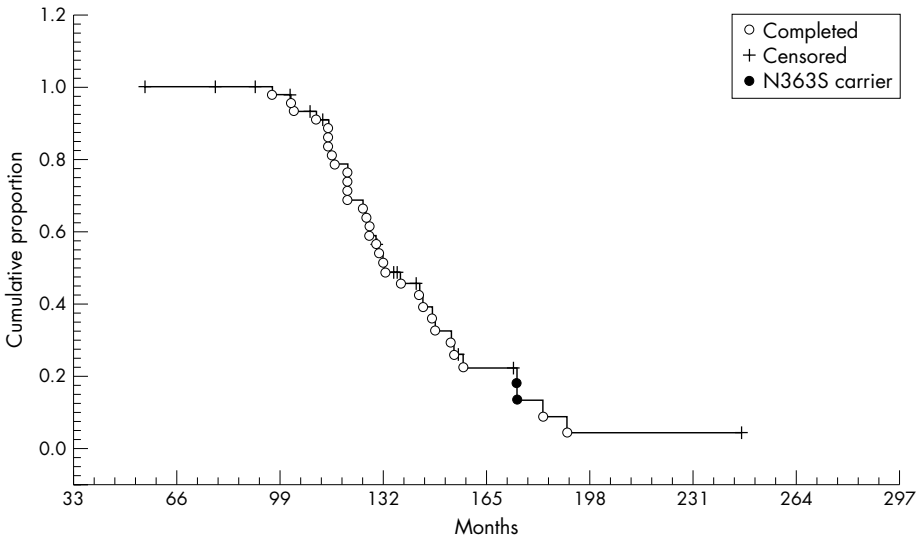

Figure 1 Kaplan-Meier proportional estimates of the number of DMD patients still ambulant at follow up (+censored: patients still ambulant).

the trial. Mean age at entry was 7.6 years (range 3.6–12 years).

Identification of *GRL* polymorphisms

PCR/SSCP/direct sequencing of the *GRL* gene was performed in 12 of the 48 DMD patients and revealed in two of them (2/12; 16%) a heterozygous 1220A>G mutation, resulting in a Asn363Ser (N363S). The 1220A>G mutation causes the loss of a *Tsp5091* restriction enzyme site. *Tsp5091* digestion in all 48 DMD patients confirmed the N363S polymorphism in the two previously identified patients and identified a third patient (3/48; 6,2%).

Steroid response

The three N363S positive DMD patients (patients 3, 15, and 41) were 5.2, 11, and 9.4 years old when they started steroid treatment. Patients 3 and 41 were on prednisone and patient 15 on deflazacort.

To detect a possible effect on DMD progression of N363S polymorphism age at loss of ambulation was evaluated in the N363S carrier and non-carrier DMD patients. Thirty three of the 48 DMD patients have lost ambulation. Age at loss of ambulation in two of the N363S carrier DMD patients was 168 months in patient 41, and 174 months in patient 15, whereas the mean age of loss of ambulation in our DMD patient cohort was 130 (SD 21.7) months. The third N363S carrier was still ambulant at last evaluation (110 months). Only two of the 45 non-carrier DMD patients (patients 9 and 19) lost ambulation at a later age (188 and 189 months) than did the two N363S carriers. Z score, right side probability, predicts that the probability of observing a patient with age at loss of ambulation greater than 168 months is $p<0.005$, and the probability of observing two consecutive patients is less than $p<0.0025$. A Kaplan-Meier curve was used to include in our analysis the ambulant DMD patients at the time of the last evaluation and it revealed that there was a less than 20% chance of being ambulant at an age greater than 168 months. Four of our DMD patients had lost ambulation later than 168 months, and of them two (50%) had the N363S polymorphism (fig 1). A Cox proportional hazard model was used to examine factors that could influence age at loss of ambulation. This analysis revealed a significant survival advantage both in the patients who started steroid therapy at a younger age ($t = -4.63$; $p = 0.00004$) and in those who had been receiving steroid therapy longest ($t = -4.63$; $p = 0.00004$). The presence of N363S polymorphism was not statistically significant ($t = 0.29$; $p = 0.76$).

No significant differences were detected between the N363S carriers and the non-carrier DMD patients in neither functional nor timed tests performed after one year of treatment.

DISCUSSION

To determine whether differences in *GRL* could be one of the molecular mechanisms underlying steroid-response in DMD, we searched for *GRL* polymorphisms in a group of 48 DMD patients treated with steroids for at least one year.

Three of the 48 DMD patients showed the N363S change in their *GRL* gene. The frequency (about 6%) of the N363S polymorphism in our DMD population is similar than previously reported. The N363S amino acid change contributes a new potential phosphorylation site and has been shown to be associated with altered sensitivity to glucocorticoids.[22] Carriers of this polymorphism showed a larger cortisol suppression, higher insulin response, and a trend towards lower age, body mass index, and sex adjusted bone mineral densitiy.[22]

To check the hypothesis that the *GRL* gene may be a candidate factor in the clinical response to exogenously administrated glucocorticoid, we looked at the age at loss of ambulation since it is a well defined parameter which tests the whole motor function and offers a relatively straightforward measure of the severity of the disease. When age at loss of ambulation is considered, a trend towards a better outcome of N363S-DMD carriers emerges: two N363S-DMD carriers had lost ambulation at a later age than the non-carrier DMD patients, while the third carrier is still ambulant. This last boy is younger than the average age at loss of ambulation in our cohort of DMD treated patients and does not allow us to draw final conclusions, however at his age about 11% of treated patients had already lost ambulation. Unfortunately, the subgroup of N363S carriers is too small to permit statistical analysis and further studies in a larger population are needed. Indeed, the Kaplan Meier curve did not achieve statistical significance even though it did clearly show a better outcome for the N363S-DMD carriers. However, when used to consider only the patients who had lost ambulation, the Z score test did reach significance which suggests that the N363S polymorphism does have a real modulating effect on steroid response.

Previous studies have suggested that the N363S polymorphism effect may be small but cumulative and become more evident over time, helping to explain the lack of short term effects of N363S polymorphism in DMD.

Our data would seem to suggest that although *GRL* polymorphisms may induce increased long term sensitivity to glucocorticoids, other mechanisms may underlie steroid action and tissue sensitivity.[23] Additional studies regarding the molecular mechanisms of glucocorticoid action and larger numbers of subjects are needed before the interactions between glucocorticoid and their receptors are fully understood.

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